Molecular Analysis of the Immunoglobulin Heavy Chain Gene in the Diagnosis of Primary Cutaneous B Cell Lymphoma

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The diagnosis of primary cutaneous B cell lymphoma can be difficult on the basis of histologic and immunophenotypic features alone. Previous polymerase chain reaction studies for detection of a clonal population in nodal B cell lymphomas have employed different primer pairs with detection sensitivities varying between 34% and 94% but there have been no comprehensive studies of primary cutaneous B cell lymphoma. We compared the sensitivity of different sets of consensus primers to amplify the CDR3 VDJ region of the immunoglobulin heavy chain gene in combination with an immunoglobulin heavy chain joining region consensus primer to detect a monoclonal population in 39 cases of primary cutaneous B cell lymphoma. Radiolabeled products were analyzed with denaturing 6% polyacrylamide gel electrophoresis. Sequence analysis was used to confirm amplification of clonal immunoglobulin heavy chain gene rearrangements and to establish whether somatic hypermutation can interfere with primer binding. Clonal immunoglobulin heavy chain gene rearrangements were demonstrated in 79% of cases (74% with leader sequences, 64% with FR1, and 45% with FR3 primers). Somatic hypermutation at primer binding sites was confirmed in cases where a false negative result was obtained with the FR3 primer. Although monoplex polymerase chain reaction amplification using the leader sequence primers is the most sensitive method for detecting a clonal population, six primers are required in six different reactions. Our findings suggest initial analysis with the FR3 primer and subsequent analysis using leader sequences in negative cases. Our data indicate that the FR3 consensus primer alone is not sufficient for a comprehensive analysis of primary cutaneous B cell lymphoma. ***Key words: polymerase chain reaction/CDR 3 VDJ region/somatic hypermutation/clonal B-cell population. J Invest Dermatol 117:984–989, 2001***

The diagnosis of primary cutaneous B cell lymphoma (PCBCL) can be difficult on the basis of histologic and immunophenotypic criteria alone (Rijlaarsdam *et al*, 1990; Ritter *et al*, 1994). Light chain restriction is not found universally in B cell lymphomas and the detection of clonal immunoglobulin gene rearrangements using southern blot analysis is time-consuming, requires relatively large amounts of fresh material (a minimum of 10 μg of DNA), and can only detect a clonal rearrangement if it comprises at least 1%–5% of the total cell population.

In contrast, the polymerase chain reaction (PCR) is a rapid, cost-effective technique that can be used to amplify unique V(D)J rearrangements of the immunoglobulin gene. It requires much smaller amounts of DNA, and may be used for analysis of archival paraffin-embedded material. The splicing of V, D, and J regions, with random insertion and deletion of nucleotides at the junctions, is responsible for the diversity of antigen recognition and forms a DNA segment of unique length (Tonegawa, 1983). Consensus primers for relatively conserved framework regions (FR1, FR2, FR3) and the JH region have been used to amplify the third complementary determining region (CDR3) by PCR. If a polyclonal lymphoid population is present, numerous variable sized copies will be produced that will appear as a smear or multiple bands following electrophoretic separation. A clonal population of B cells will produce a single discrete product.

Previous PCR studies for the detection of a clonal population in systemic B cell lymphomas have employed a variety of different primer pairs and have reported sensitivities as high as 94% (McCarthy *et al*, 1990; Trainor *et al*, 1990; 1991; Deane and Norton, 1991; Deane *et al*, 1991; Ramasamy *et al*, 1992; Diss *et al*, 1993; Segal *et al*, 1994a, b; Aubin *et al*, 1995; Lombardo *et al*, 1996; Essop *et al*, 1997; Derkson *et al*, 1999). A recent study of PCBCL employing an FR3 primer, however, found a detection sensitivity of only 34% (Ritter *et al*, 1997). This lower sensitivity may reflect somatic hypermutation of the VH region, which results in primer mismatching and failure to amplify the clonal population of B cells (Diss *et al*, 1993; Segal *et al*, 1994b; Aubin *et al*, 1995; Lombardo *et al*, 1996). Follicle center cell lymphoma, marginal zone lymphoma, and high-grade large cell lymphomas of extra-nodal origin exhibit high degrees of somatic hypermutation, which may explain the lower detection sensitivity in these types of lymphoma compared to chronic lymphocytic leukemia (B-CLL) and mantle zone lymphomas (Deane and Norton, 1991; Segal *et al*, 1994b; Lombardo *et al*, 1996; Essop *et al*, 1997).

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Abbreviations: B-CLL, chronic lymphocytic leukemia; IgH, immunoglobulin heavy chain; PCBCL, primary cutaneous B cell lymphoma.

In view of the expected high rate of somatic hypermutation occurring in PCBCL we undertook this study to compare the sensitivity of a number of different consensus primer pairs for the detection of clonal immunoglobulin heavy chain (IgH) gene rearrangements in proven PCBCL samples.

MATERIALS AND METHODS

Frozen tissue samples from 39 cases of PCBCL were retrieved. The corresponding histologic and immunohistochemistry sections were reviewed by three of the authors (FJC, EC, RRJ) and classified according to the REAL classification (Harris *et al*, 1994). The diagnosis of PCBCL was based on diagnostic or compatible histology plus either light chain restriction or evidence of a clonal B cell population using southern blot IgH gene analysis. In all cases there had been no evidence of extra-cutaneous disease at presentation or for 6 mo following diagnosis, assessed by adequate staging procedures, which included a complete physical examination, thoraco-abdominal-pelvic CT scan, and bone marrow biopsy.

Immunohistochemical staining Detailed immunophenotyping was performed on routinely fixed paraffin-embedded tissue sections using a panel of monoclonal antibodies that included anti-CD3, anti-CD20, anti-CD21, anti-CD79a, anti-κ, and anti-λ, and the monoclonal anti-*bcl-2* protein (Dako, High Wycombe, U.K.).

DNA extraction DNA was extracted from fresh skin biopsy specimens according to standard procedures by proteinase K digestion and phenol/chloroform extraction. Control DNA samples were extracted from the Daudi cell line (monoclonal control) and from six cases of benign cutaneous lymphoid hyperplasia (polyclonal controls). DNA was also extracted from involved skin of four cases of systemic B cell lymphoma that had presented with cutaneous lesions.

Southern blot analysis DNA was digested separately with restriction enzymes *Eco* R1 and *Hind* III and subjected to southern blot analysis. Filters were hybridized with radiolabeled probes under high stringency conditions as previously described (Foroni *et al*, 1984), using a genomic DNA probe for the JH region of the IgH gene.

IgH gene PCR amplification Three different sets of consensus primers were used to amplify the CDR3 VDJ region of the IgH gene: (i) a V region consensus primer corresponding to the third framework region (FR3) (Deane and Norton, 1990a) in combination with a JH consensus primer (Deane and Norton, 1990a); (ii) a series of seven V region consensus primers corresponding to each FR1 region family (Deane and Norton, 1990b) in combination with a JH consensus primer (each V region primer was used in a separate monoplex reaction); (iii) a series of six consensus primers corresponding to the leader segment 5′ to each VH region family (Campbell *et al*, 1992) in combination with a JH consensus primer (each leader segment primer was used in a monoplex reaction). The leader sequence primers are slightly longer than those published because this modification has produced more effective amplification.

In eight cases in which the V region/JH consensus primers had failed to detect a clonal IgH gene rearrangement, repeat analysis with V region primers and a mix of JH primers (JH1, JH2, JH4, JH5 (JH consensus), JH3, and JH6) was performed. Rarely, JH gene segments are not recognized by the JH consensus primer. False negative results may also occur due to somatic hypermutation in the JH region (Aubin *et al*, 1995).

Figure 1 shows a schematic representation of the rearranged IgH gene and the relative positions of the amplification primers used in the three different strategies.

The reaction mix (20 μl final volume) consisted of 1 μg template DNA, 1 × PCR buffer (Amersham Pharmacia Biotech, Little Chalfont, U.K.), 2.5 mM $MgCl_2$, 200 μM [α^{33}P]dCTP, dNTPs, 0.01% Tween 20, 200 nM synthetic oligonucleotide consensus primers (Amersham Pharmacia Biotech) for the JH region and either FR3, FR1, or leader sequence regions, and 1 unit of Taq Polymerase (Amersham Pharmacia Biotech) in combination with *taq* start antibody (Clontech, Basingstoke, U.K.). A negative control reaction containing reaction mix but no template DNA was examined for each PCR assay performed. All PCR reactions were performed in duplicate for each DNA sample.

Monoplex PCR was performed using a Perkin Elmer thermal cycler (model 9700, Warrington, U.K.). The annealing temperatures used were as follows: FR3 primer, 58°C; FR1 primers, 60°C–62°C; leader sequence primers, 65°C–66°C. The PCR reactions were performed over 30–33 cycles.

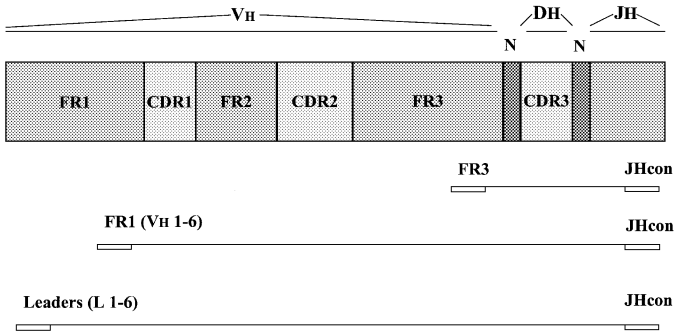

Figure 1. Schematic representation of the rearranged IgH gene and the relative positions of the amplification primers used in the three different strategies.

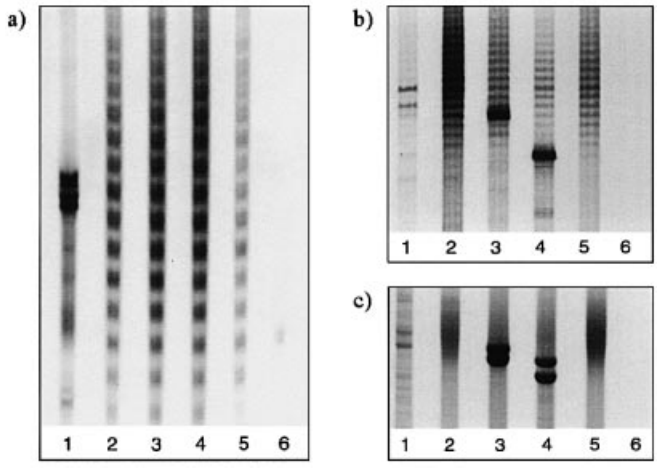

Figure 2. Composite figure illustrating autoradiographs of the IgH gene fingerprinting analysis using the different amplification strategies. (*a*) FR3 amplification strategy. (*b*) FR1 amplification strategy (consensus primer for the VH3 family). (*c*) Leader sequence amplification strategy (consensus primer for the VH3 family). *Lane 1*, a clonal rearrangement is identified with all three amplification strategies in a patient with a high-grade PCBCL. *Lane 2*, polyclonal amplification is demonstrated in DNA from a low-grade PCBCL. *Lanes 3*, *4*, a clonal rearrangement is seen with the FR1 and leader sequence primers but not the FR3 primer in two cases of low-grade PCBCL. *Lane 5* demonstrates a polyclonal ladder (obtained with all primers) from a patient with a reactive B cell infiltrate. *Lane 6*, negative control (reaction mix but no template DNA).

The PCR products with appropriate molecular weight markers were loaded onto 2% agarose gels, subjected to electrophoresis at 100 V, and stained with ethidium bromide. If amplification was successful, an aliquot of the radiolabeled PCR products was run on denaturing 6% polyacrylamide gels. Formamide (95% formamide, 20 mmol per l ethylenediamine tetraacetic acid, 0.05% xylene cyanol FF, 0.05% bromophenol blue) loading dye was added to each amplified product and denatured at 94°C for 5 min. The samples were put on ice prior to loading onto denaturing polyacrylamide gels [6% acrylamide (Gibco BRL, Paisley, U.K.), 7 M urea (United States Biochemicals, Cleveland, OH)]. The gel was run at 30 W for 2–7 h, depending on the size of the PCR product, at room temperature in 0.5 × TBE buffer. Autoradiography was carried out at room temperature for 24–72 h with an intensifying screen (Appligene Oncor, Watford, U.K.).

Sequence analysis Sequence analysis was performed on selected products to confirm amplification of a clonal immunoglobulin rearrangement and to identify whether mutations at FR3 primer binding sites had occurred in cases in which a clonal rearrangement was demonstrated with leader or FR1 primers but in which a polyclonal result was obtained with the FR3 primer. Clonal bands were excised

Table I. Proportion of clonal IgH chain gene rearrangements obtained in cases of PCBCL, systemic B cell lymphoma and benign cutaneous lymphoid hyperplasia using leader sequences and FR1 and FR3 primers

PCBCL	Leaders	FR1	FR3	Total
Total	29/39 (74%)	25/39 (64%)	17/38 (45%)	31/39 (79%)
High-grade	**8/10 (80%)**	**6/10 (60%)**	**7/10 (70%)**	**9/10 (90%)**
Low-grade	**21/29 (72%)**	**19/29 (66%)**	**10/28 (36%)**	**22/29 (76%)**
Systemic B cell lymphoma	3/4 (75%)	3/4 (75%)	2/4 (50%)	4/4 (100%)
Benign cutaneous lymphoid hyperplasia	0/6	0/6	0/6	0/6

Table II. Sequence analysis of the FR3 region corresponding to the FR3 primer binding site in nine cases of PCBCL[a]

Patient	Diagnosis	VH Family	Closest germline gene	FR3 Clone?	Sequence	No. of mutations
1	MZL	6	V6-01 (DP 74)	No	AC ACG GCT GT**T** TAT TA**T** TGT	2
2	MZL	1	V1-69 (DP10)	No	AC ACG GCC GT**A** TA**C** TA**T** **G**G**C**	5
3	MZL	1	V1-08 (DP 15)	No	AC ACG GCC GT**C** TAT **G**AC TGT	2
4	MZL	3	V3-15 (DP38)	No	AC ACA GCC GT**A** TAT **ATT GCA**	7
5	FCL	1	V1-46 (DP7)	No	AC ACG GCC **A**TG TAT TA**T** TGT	2
6	FCL	4	V4-34 (DP63)	No	AC ACG GCT **AT**C TAT TA**T** TG**C**	4
7	MZL	3	V3 (1–17)	Yes	AC ACG GCT GT**G** TAT TAC TGT	1
8	MZL	1	V1-18 (DP14)	Yes	AC ACG GCC GTG TAT TAC TGT	0
9	MZL	1	V1-69 (DP10)	Yes	AC ACG GCC CT**A** TAT TAC TGT	1

[a]MZL, marginal zone lymphoma; FCL, follicle center lymphoma nucleotides highlighted in bold = point mutations (when compared with consensus FR3 primer sequence).

from the gel and the product was re-amplified using a biotinylated JH consensus primer together with the appropriate 5′ primer. Amplification was determined by electrophoresis and the remaining PCR product was passed through a microspin column (Amersham Pharmacia Biotech) to remove unincorporated primers. The amplification products were then immobilized on the surface of streptavidin-coated microbeads (Dynabeads M-280, Dynal, Oslo, Norway). The sequenase chain termination DNA sequencing method was performed using the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemicals). Products were analyzed by denaturing polyacrylamide gel electrophoresis and visualized by autoradiography.

Sensitivity of technique To determine the sensitivity of our technique, DNA obtained from the Daudi B cell line (ECACC no. 89120702) (known to harbour a clonal VH3 family IgH gene rearrangement) was serially diluted with DNA obtained from a polyclonal control. PCR/denaturing polyacrylamide gel electrophoresis was performed using the FR1/VH3 primer in combination with the JH consensus primer.

RESULTS

Histologic diagnosis of lymphoma Of the 39 cases that made up the study group, 10 were classified as diffuse large cell lymphoma (high-grade) and 29 were low-grade PCBCL (21 marginal zone lymphoma, five follicle center lymphoma, three definite lymphoma but difficult to classify). The high-grade lymphomas and all but seven low-grade lymphomas showed diagnostic histology. Of the seven cases in which histology was compatible but not diagnostic, three showed a clonal IgH population by southern blot analysis and the remaining four cases demonstrated light chain restriction.

Demonstration of monoclonality using the different consensus primer pairs (Table I)

*FR3 + JH consensus primers (**Fig 2a**)* Seventeen of 38 cases (45%) demonstrated a monoclonal rearrangement of the IgH gene. The group consisted of seven out of 10 (70%) high-grade lymphomas but only 10 of 28 (36%) low-grade lymphomas. Only 38 cases were analyzed using the FR3 primer as DNA was not available in one case.

*FR1 + JH consensus primers (**Fig 2b**)* Twenty-five out of 39 (64%) showed a monoclonal rearrangement using the FR1 series of consensus primers in combination with the JH consensus primer. This included six of 10 (60%) high-grade lesions and 19 out of 29 (66%) low-grade lymphomas.

*Leader sequences + JH consensus primers (**Fig 2c**)* Twenty-nine of 39 (74%) showed a monoclonal rearrangement using the series of leader sequence consensus primers in combination with the JH primer, consisting of eight out of 10 (80%) high-grade and 21 of 29 (72%) low-grade lymphomas.

Overall, a clonal IgH gene rearrangement was found in 31 of 39 (79%) PCBCL, which included nine of 10 (90%) high-grade and 22 of 29 (76%) low-grade lymphomas. In 14 cases (37%) a clonal rearrangement was identified with all three primer sets, and in six cases (15%) a clone was detected with only one primer set (five with the leader sequences and one with the FR3 primer). The FR1 primer set did not identify a clonal rearrangement in any cases where a clone was not detected with either the FR3 or leader sequence primers. In eight cases where no clonal IgH gene rearrangement was detected, repeat analysis with a mix of JH primers failed to detect any further clonal rearrangements.

A clonal IgH rearrangement was detected in all four cases of systemic B cell lymphoma with cutaneous presentation (three follicle center lymphoma, one unclassifiable), but with all three primer sets in only one case. In two cases a clone was detected with two primer sets (FR3 and FR1 in one case and FR1 and leader sequences in the other). In the final patient an IgH clone was detected with the leader sequences alone.

The six cases of reactive cutaneous lymphoid hyperplasia produced a polyclonal result with all three primer sets.

The sensitivity of this technique for the detection of a clonal IgH gene rearrangement was approximately 0.5% when DNA from the Daudi cell line (VH3 family monoclonal control) was serially diluted with DNA from a reactive B cell infiltrate.

Table III. Comparison of the published methods and detection sensitivities for clonal rearrangement of the IgH chain gene[a]

Reference	Lymphoma type	Gold standard	PCR type primer pairs	Product visualization	Sequence confirmation	Detection sensitivity
PCR products analysed on agarose gels						
Trainor *et al* (1990)	14 B-NHL, 9 B-CLL	immuno/histology, SBA	single round FR3/LJH or VLJH	ethidium bromide	no	19/23 (83%)
Deane *et al* (1991)	ALL, B-CLL	unique band with monoplex FR1 primers	single round* FR1/JHcon	ethidium bromide	yes	15/16 (94%)
Ramasamy *et al* (1992)	9 ALL, 7 NHL, 4 CLL, 2 MM	SBA	seminested FR2/JH primers	ethidium bromide	yes	18/22 (82%)
Segal *et al* (1994a)	43 CLL, 57 SLL, 32 MCL, 17 plasma cell, 7 hairy cell	immuno/histology	single round FR1,FR3/JHcon	ethidium bromide	no	FR1131/156 (84%) FR3150/156 (96%)
Segal *et al* (1994b)	FCCL	immuno/histology	single round FR1,FR3/JHcon	ethidium bromide	no	FR1 27/60 (45%) FR3 31/60 (52%)
Lombardo *et al* (1996)	intermediate & high-grade NHL	immuno/histology	single round FR1,FR3/JHcon	ethidium bromide	no	FR3 43/71 (61%), FR1 11/15 of those -ve with FR3
Essop *et al* (1997)	38 low-grade, 31 high-grade 1LP HD	immuno/histology	seminested FR2,FR3/ JH primers	ethidium bromide	no	65/70 (93%) FR2 58%, FR3 77%
PCR products analysed on polyacrylamide gels						
McCarthy *et al* (1990)	3 FCCL, 4 non-FCCL B-NHL, 2 B-CLL	SBA	single round VH/JH primers	ethidium bromide	no	7/9 (78%)
Trainor *et al* (1991)	12 B-ALL, 9 B-CLL, 21 B-NHL, 10 MM	SBA	seminested FR3/JH primers	ethidium bromide	no	39/52 (75%)
Deane *et al* (1991)	61 ALL, 55 B-CLL & B-NHL	immuno/histology	single round FR1/JHcon	SBA with VH specific probes	no	109/116 (94%)
Diss *et al* (1993)	47 MALT, 23 CB/CC, 11 B-CLL, 6 MCL, 4 LPL, 3 splenic MZL	SBA	seminested FR2/JH & FR3/JH primers	ethidium bromide	no	85/94 (90%) FR2 85%, FR3 80%
Aubin *et al* (1995)	9 high-grade, 8 FCCL, 9 MCL, 4 non-FCCL, non-MCL	immuno/histology	single round FR3/JHcon or mix FR1f/JH primers FR1c/JH primers FR2,5,6/JH primers	ethidium bromide	no	19/30 (63%) 18/30 (60%) 18/28 (64%) 22/28 (79%)
Ritter *et al* (1997)	small cell & large cell PCBCL	immuno/histology	seminested FR3/JH primers	ethidium bromide	no	12/35 (34%)
Derkson *et al* (1999)	18 B-CLL, 7 MCL, 20 FCCL, 5 MALT, 18 DLCL, 1 BL, 1 MM	SBA	single round FR3/JH primers FR2/JH primers FR1/JH primers	ethidium bromide radiolabeled HRF fluorescent GS radiolabeled HRF ethidium bromide	no	42/70 (60%) 45/70 (64%) 48/70 (69%) 54/70 (77%) 45/70 (64%)

[a]NHL, non-Hodgkin's lymphoma; CLL, chronic lymphocytic leukemia; ALL, acute lymphoblastic leukemia; MM, multiple myeloma; FCCL, follicle center cell lymphoma; LP HD, lymphocyte predominant Hodgkin's disease; MALT, mucosa associated lymphoid tissue; CB/CC, centroblastic centrocytic; MCL, mantle zone lymphoma; LPL, lyphoplasmacytoid lymphoma; MZL, marginal zone lymphoma; DLCL, diffuse large cell lymphoma; BL, Burkitt's lymphoma; SLL, small lymphocytic lymphoma; *multiplex analysis. All other studies employed primers in a monoplex reaction.

Comparison with southern blot analysis There were 16 patients with PCBCL showing diagnostic histology that had previously undergone southern blot analysis. Of the 12 patients who had a southern blot clonal rearrangement of the IgH chain gene, 11 were clonal by PCR and one was polyclonal. In the four patients with germline southern blot analysis, two were clonal by PCR and two were polyclonal (these patients had immunophenotypic evidence of light chain restriction).

Sequence analysis Sequence analysis of clonal rearrangements was performed in selected cases to confirm amplification of the correct IgH VDJ sequence and to determine whether somatic hypermutation of the IgH gene was the cause of false negative results. Discrete bands representing clonal IgH rearrangements obtained using FR1 or leader sequence primers were sequenced in nine cases (six with a polyclonal pattern and three with a clonal pattern using the FR3 primer). Sequences were compared with germline sequences using the Vbase database (Cook and Tomlinson, 1995) and DNAplot (Giudicelli *et al*, 1997) (http://www.mrc-cpe.cam.ac.uk/imt-doc).

Point mutations of the sequence corresponding to the FR3 primer were identified in the six cases where a polyclonal pattern had been obtained. The number of mutations varied between two and seven and in three cases involved the nucleotide at the 3′ end of the sequence. In contrast, the three cases sequenced in which a clonal rearrangement had been identified showed either a single point mutation (two cases) or no mutations (one case) of the FR3 primer binding site (**Table II**).

DISCUSSION

The detection of clonal IgH gene rearrangements using either southern blot analysis or PCR is a valuable method for assessing clonality in cutaneous B cell infiltrates where histologic and immunophenotypic features are not diagnostic (Landa *et al*, 1993). To obtain a correct diagnosis, however, highly sensitive and

specific PCR methods are required. In this study we used a monoplex PCR method incorporating three different sets of IgH V region consensus primers in conjunction with an IgH J region consensus primer to assess clonality in a set of DNA samples obtained from lesions of PCBCL.

Overall, with this PCR methodology, we demonstrated that 31 of the 39 (79%) PCBCL showed a clonal IgH gene rearrangement. We were able to detect a clonal IgH rearrangement in nine of 10 (90%) high-grade and 22 of 29 (76%) low-grade PCBCL. Our results suggest that use of the leader sequence primers was the most sensitive detection method. Primers for the FR1 and FR3 regions were less sensitive, but were able to detect a small number of IgH clonal rearrangements that were not detected using the leader sequence primers. There were no cases, however, in which only the FR1 primers detected an IgH clone. There were only eight cases of low-grade PCBCL in which all three different primer sets detected a clonal rearrangement.

A clonal IgH gene rearrangement was not detected in 21% of PCBCL with any of the primer sets used. Clonal IgH gene rearrangements may be undetectable, or detected with some primer pairs but not others, because of somatic hypermutation of rearranged VH gene segments, which may prevent annealing of the FR primers and to a lesser extent the JH primer (Diss *et al*, 1993; Segal *et al*, 1994b; Aubin *et al*, 1995; Lombardo *et al*, 1996). The primers will anneal to DNA from the reactive B cell population, however, resulting in amplification of the polyclonal population alone. Cutaneous B cell lymphomas may also often consist of a large number of reactive B cells that can reduce the signal intensity of the monoclonal population, and a very small B cell clone may therefore be below the threshold for detection (Trainor *et al*, 1991; Ritter *et al*, 1997).

We were able to demonstrate somatic hypermutation at primer binding sites by sequence analysis of six selected cases in which clonal rearrangements were detected with leader or FR1 primers but not the FR3 primer. Between two and seven point mutations were detected at the FR3 primer binding site. In contrast, three cases in which a clone was detected using the FR3 primer had either a single or no point mutation at the same site.

Comparison of the results obtained in our study with the southern blot analysis data, which were used as one of the ''gold standards'' for the diagnosis of PCBCL, demonstrated that 11 of 12 samples with a detectable southern blot rearrangement were clonal by PCR. The detection of a clonal rearrangement using southern blot analysis is not affected by somatic hypermutation, which is likely to account for the one negative PCR sample. Interestingly, of the four patients with germline southern blot analysis but diagnostic histology and immunophenotypic features, two were clonal by PCR techniques, confirming that PCR methods are more sensitive. Two cases with evidence of light chain restriction did not demonstrate clonality either by southern blot analysis or PCR methodology, however, presumably reflecting a lack of sensitivity and somatic hypermutation, respectively.

There are few previous PCR studies analysing the IgH gene in PCBCL. In 1997, Ritter *et al* published data on 43 cases of PCBCL (23 small cell, 15 large cell) using the FR3 primer alone. A clonal IgH rearrangement was demonstrated in seven of 23 (30%) with small cell morphology and five of 12 (42%) with large cell morphology. Overall, these FR3 primer results are similar to our findings. This study was performed on paraffin-embedded tissue, and DNA degradation may have prevented amplification of the IgH gene in 30% of cases because only 70% of cases demonstrated an amplifiable β-globin gene. More recently Nihal *et al* compared IgH PCR assays using multiplex FR1 and FR3 primers and DNA extracted from fresh tissue in 12 cases of cutaneous B cell lymphoma (nine PCBCL). In contrast to our findings a clonal IgH gene rearrangement was detected in 11 of 12 cases with the FR3 primer but only five of 12 cases with the FR1 primer set (Nihal *et al*, 2000).

Previous studies (reviewed in **Table III**) have used different sets of primers for detection of clonal IgH gene rearrangements in systemic B cell lymphoma (McCarthy *et al*, 1990; Trainor *et al*, 1990, 1991; Deane and Norton, 1991; Deane *et al*, 1991; Ramasamy *et al*, 1992; Diss *et al*, 1993; Segal *et al*, 1994a, b; Aubin *et al*, 1995; Lombardo *et al*, 1996; Essop *et al*, 1997; Derkson *et al*, 1999) and the majority employ primers for the FR3 region (Trainor *et al*, 1990; 1991; Diss *et al*, 1993; Segal *et al*, 1994a, b; Aubin *et al*, 1995; Lombardo *et al*, 1996; Essop *et al*, 1997; Derkson *et al*, 1999). Some have compared results obtained using a consensus FR3 primer with those obtained using FR1 (Segal *et al*, 1994a, b; Aubin *et al*, 1995; Lombardo *et al*, 1996; Derkson *et al*, 1999) or FR2 (Diss *et al*, 1993; Essop *et al*, 1997; Derkson *et al*, 1999) consensus primers and have shown variable rates of detection, but when the results obtained with different primer sets are combined, the overall detection sensitivity is increased (Diss *et al*, 1993; Segal *et al*, 1994a, b; Lombardo *et al*, 1996; Essop *et al*, 1997; Derkson *et al*, 1999), which concurs with our results. The detection sensitivities are much lower in those lymphomas derived from germinal center and post germinal center B cells (MALT, follicle center cell, and high-grade diffuse large cell lymphomas) that exhibit high degrees of somatic hypermutation (Diss *et al*, 1993; Segal *et al*, 1994b; Aubin *et al*, 1995; Lombardo *et al*, 1996; Essop *et al*, 1997; Ritter *et al*, 1997; Derkson *et al*, 1999).

Various PCR methods for analysis of the IgH gene have been published. Many studies employ semi-nested PCR techniques (Trainor *et al*, 1991; Ramasamy *et al*, 1992; Diss *et al*, 1993; Essop *et al*, 1997; Ritter *et al*, 1997) to increase sensitivity but specificity can be reduced. We used a single-round monoplex PCR reaction to increase specificity in combination with a highly sensitive electrophoretic technique. Some studies have only used agarose gels for analysis of PCR products (Trainor *et al*, 1990; Deane *et al*, 1991; Ramasamy *et al*, 1992; Segal *et al*, 1994a, b; Lombardo *et al*, 1996; Essop *et al*, 1997) (**Table III**). Although we used this method to confirm PCR amplification of the correct product, it was impossible to distinguish a monoclonal from a polyclonal product in all but four samples. In other studies nondenaturing polyacrylamide gel electrophoresis with ethidium bromide staining has been used (McCarthy *et al*, 1990; Trainor *et al*, 1991; Diss *et al*, 1993; Aubin *et al*, 1995; Ritter *et al*, 1997) with a sensitivity of approximately 1%–10% (Aubin *et al*, 1995). Although more time-consuming, radioactively labeled nucleotides and denaturing polyacrylamide gel electrophoresis increases the sensitivity to 0.1%–1% (Deane and Norton, 1991; Rosenquist *et al*, 1999) as in our study. An increased sensitivity is important in PCBCL because the clonal population may be very small. We used a modification of the method employed by Deane *et al* (Deane and Norton, 1991; Chim *et al*, 1996), which produces a fingerprint from polyclonal DNA against which a clonal rearrangement can be detected as a discrete band. The polyclonal fingerprint pattern serves as a convenient internal control for monitoring the efficacy of the PCR reaction and this confirmed the presence of amplifiable DNA in all 39 of our cases.

Analysis of fluorescent PCR products on automated sequencers has a similar sensitivity (10^{-2}–10^{-3}) (Landman-Parker *et al*, 1996) to our method and is increasingly replacing other methods of detection. Recently, Derkson *et al* (1999) compared different PCR-based approaches to assess clonal IgH gene rearrangements in B cell neoplasia. They used FR3/JH consensus primers and compared radiolabeled high resolution fingerprinting with non-denaturing polyacrylamide gel heteroduplex analysis and fluorescently labeled gene scanning using an automated sequencer. A clone was detected in 69% with gene scanning compared with 64% with high resolution fingerprinting and 60% with heteroduplex analysis, suggesting that gene scanning of fluorescent PCR products and automated sequence analysis is the most sensitive method of detection, but the difference was not statistically significant.

In summary, we have been able to detect a monoclonal B cell population in 79% of PCBCL using monoplex radiolabeled PCR and three different sets of consensus primers to amplify the IgH gene with high resolution fingerprint analysis. The leader sequence primers provided the greatest sensitivity. Sequence analysis con-

firmed hypermutation of FR3 primer binding sequences in cases that produced a false negative result with the FR3 primer. For diagnostic purposes, initial analysis with the FR3 primer may identify a clonal IgH rearrangement but, in cases that prove negative, consensus primers for the leader sequences must be employed.

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